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TITLE

<u>DIPEPTIDYL PEPTIDASES</u>

FIELD OF INVENTION

5   The invention relates to a dipeptidyl peptidase, to a
nucleic acid molecule which encodes it, and to uses of the
peptidase.

BACKGROUND OF THE INVENTION

10   The dipeptidyl peptidase (DPP) IV-like gene family is a
family of molecules which have related protein structure
and function [1-3]. The gene family includes the
following molecules: DPPIV (CD26), dipeptidyl amino-
peptidase-like protein (DPP6) and fibroblast activation
15   protein (FAP) [1,2,4,5]. Another possible member is
DPPIV-β[6].

The molecules of the DPPIV-like gene family are serine
proteases, they are members of the peptidase family S9b,
20   and together with prolyl endopeptidase (S9a) and
acylaminoacyl peptidase (S9c), they are comprised in the
prolyl oligopeptidase family[5,7].

DPPIV and FAP both have similar postproline dipeptidyl
25   amino peptidase activity, however, unlike DPPIV, FAP also
has gelatinase activity[8,9].

DPPIV substrates include chemokines such as RANTES,
eotaxin, macrophage-derived chemokine and stromal-cell-
30   derived factor 1; growth factors such as glucagon and
glucagon-like peptides 1 and 2; neuropeptides including
neuropeptide Y and substance P; and vasoactive
peptides[10-12].

35   DPPIV and FAP also have non-catalytic activity; DPPIV
binds adenosine deaminase, and FAP binds to $\alpha_3\beta_1$ and $\alpha_5\beta_1$
integrin[13-14].

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In view of the above activities, the DPPIV-like family
members are likely to have roles in intestinal and renal
handling of proline containing peptides, cell adhesion,
peptide metabolism, including metabolism of cytokines,
5   neuropeptides, growth factors and chemokines, and
immunological processes, specifically T cell
stimulation[3,11,12].

Consequently, the DPPIV-like family members are likely to
10   be involved in the pathology of disease, including for
example, tumour growth and biology, type II diabetes,
cirrhosis, autoimmunity, graft rejection and HIV
infection[3,15-18].

15   Inhibitors of DPPIV have been shown to suppress arthritis,
and to prolong cardiac allograft survival in animal models
in vivo[19,20].   Some DPPIV inhibitors are reported to
inhibit HIV infection[21].   It is anticipated that DPPIV
inhibitors will be useful in other therapeutic
20   applications including treating diarrhoea, growth hormone
deficiency, lowering glucose levels in non insulin
dependent diabetes mellitus and other disorders involving
glucose intolerance, enhancing mucosal regeneration and as
immunosuppressants[3,21-24].
25
There is a need to identify members of the DPPIV-like gene
family as this will allow the identification of
inhibitor(s, with specificity for particular family
member(s) which can then be administered for the purpose
30   of treatment of disease.   Alternatively, the identified
member may of itself be useful for the treatment of
disease.

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SUMMARY OF THE INVENTION

The present invention seeks to address the above
identified need and in a first aspect provides a peptide
which comprises the amino acid sequence shown in SEQ ID
5   NO:1.

This peptide has substrate specificity for the following
compounds: H-Ala-Pro-pNA, H-Gly-Pro-pNA and H-Arg-Pro-pNA.
Therefore, it is a prolyl oligopeptidase and a dipeptidyl
10   peptidase, because it is capable of hydrolysing the
peptide bond C-terminal to proline in each of these
compounds.

The peptide is homologous with human DPPIV, and
15   importantly, identity between the sequences of DPPIV and
SEQ ID NO: 1 is observed at the region of DPPIV containing
the catalytic triad residues and the two glutamate
residues of the β-propeller domain essential for DPPIV
enzyme activity. The observation of amino acid sequence
20   homology means that the peptide which has the amino acid
sequence shown in SEQ ID NO:1 is a member of the DPPIV-
like gene family. Accordingly the peptide was
provisionally named DPPIVL1, and is now named and
described herein as DPP8.
25

The following sequences of the human DPPIV amino acid
sequence are important for the catalytic activity of
DPPIV: (i) Tyr^{662} GlyTrpSerTyrGlyGlyTyrVal; (ii)
Ala^{707}AspAspAsnValHisPhe; (iii) Gly^{738}AspHisGlyIleAlaGln;
30   and (iv) Tyr^{201}ValTyrGluGluGluVal [25-28]. As described
herein, the alignment of the following sequences of DPP8:
His^{736}GlyTrpSerTyrGlyGlyTyrLeu; Leu^{816}AspGluAsnValHisPheAla;
Glu^{847}ArgHisSerIleArg and Phe^{255}ValLeuGlnGluGluPhe with
sequences (i) to (iv) above, respectively, suggests that
35   these sequences of DPP8 are likely to confer the catalytic
activity of DPP8. Thus, in a second aspect, the invention
provides a peptide comprising the following amino acid

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sequences: His[736]GlyTrpSerTyrGlyGlyTyrLeu;
Leu[816]AspGluAsnValHisPheAlaHis; Glu[847]ArgHisSerIleArg and
Phe[255]ValLeuGlnGluGluPhe; which has the substrate
specificity of the sequence shown in SEQ ID NO:1.

5

Also described herein, using multiple sequence alignment,
it is observed that DPP8 has 55% amino acid similarity and
32% amino acid identity with a *C. elegans* protein.
Further, as shown herein, a nucleic acid molecule which

10   encodes DPP8, is capable of hybridising specifically with
DPP8 sequences derived from non-human species. Together
these data suggest that DPP8 is expressed in non-human
species. Thus in a third aspect, the invention provides a
peptide which has at least 60% amino acid identity with

15   the amino acid sequence shown in SEQ ID NO:1, and which
has the substrate specificity of the sequence shown in SEQ
ID NO:1. Preferably, the amino acid identity is 75%.
More preferably, the amino acid identity is 95%. Amino
acid identity is calculated using GAP software [GCG

20   Version 8, Genetics Computer Group, Madison, WI, USA] as
described further herein. Typically, the non-human DPP8
comprises the following sequences:
His[736]GlyTrpSerTyrGlyGlyTyrLeu;
Leu[816]AspGluAsnValHisPheAlaHis; Glu[847]ArgHisSerIleArg and

25   Phe[255]ValLeuGlnGluGluPhe.

In view of the homology between DPPIV and DPP8 amino acid
sequences, it is expected that these sequences will have
similar tertiary structure. This means that the tertiary

30   structure of DPP8 is likely to include the seven-blade β
propeller domain and the α/β hydrolase domain of DPPIV.
These structures in DPP8 are likely to be conferred by the
regions comprising β-propeller, Gly[189] to Asp[606], α/β
hydrolase, Ser[607] to Ile[882] and about 70 to 100 residues in

35   the region Arg[39] to Gln[179]. As it is known that the β-
propeller domain regulates proteolysis mediated by the
catalytic triad in the α/β hydrolase domain of prolyl

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oligopeptidase, [29] it is expected that truncated forms
of DPP8 can be produced, which have the substrate
specificity of the sequence shown in SEQ ID NO:1,
comprising the regions referred to above

5   (His^{736}GlyTrpSerTyrGlyGlyTyrLeu;
Leu^{816}AspGluAsnValHisPheAlaHis; Glu^{847}ArgHisSerIleArg and
Phe^{255}ValLeuGlnGluGluPhe) which confer the catalytic
specificity of DPP8. Examples of truncated forms of DPP8
which might be prepared are those in which the region

10   conferring the β-propeller domain and the α/β hydrolase
domain are spliced together. Other examples of truncated
forms include those which are encoded by splice variants
of DPP8 mRNA. Thus although, as described herein, the
biochemical characterisation of DPP8 shows that DPP8

15   consists of 882 amino acids and has a molecular weight of
about 100kDa, it is recognised that truncated forms of
DPP8 which have the substrate specificity of the sequence
shown in SEQ ID NO:1, may be prepared using standard
techniques [30,31]. Thus in a fourth aspect, the

20   invention provides a fragment of the sequence shown in SEQ
ID NO: 1, which has the substrate specificity of the
sequence shown in SEQ ID NO:1. Preferably, the fragment
has an amino acid sequence shown in SEQ ID NO: 3, 5 or 7.

25   As described herein, the sequence shown in SEQ ID NO:1
does not contain a consensus sequence for N-linked
glycosylation. Therefore it is unlikely that DPP8 is
associated with N-linked glycosylation. In this regard,
DPP8 is distinguished from other DPPIV-like gene family

30   members, which contain between 6 and 9 consensus sequences
for N-linked glycosylation. Thus in one embodiment, an
asparagine residue in the peptide of the first aspect of
the invention is not linked to a carbohydrate molecule.

35   The analysis of DPP8 expression described herein shows
that it is likely that DPP8 is expressed as a cytoplasmic
protein. The expression of DPP8 is therefore

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distinguished from other DPPIV-like gene family members, which are expressed on the cytoplasmic membrane, or in other words, the cell surface membrane. Thus in another embodiment, the peptide of the first aspect of the
5   invention is not expressed on a cell surface membrane of a cell.

It is recognised that DPP8 may be fused, or in other words, linked to a further amino acid sequence, to form a
10   fusion protein which has the substrate specificity of the sequence shown in SEQ ID NO:1. An example of a fusion protein is described herein which comprises the sequence shown in SEQ ID NO:1 which is linked to a further amino acid sequence: a "tag" sequence which consists of an amino
15   acid sequence encoding the V5 epitope and a His tag. An example of another further amino acid sequence which may be linked with DPP8 is a glutathione S transferase (GST) domain [30]. Another example of a further amino acid sequence is a portion of CD8α [8]. Thus in one aspect, the
20   invention provides a fusion protein comprising the amino acid sequence shown in SEQ ID NO:1 linked with a further amino acid sequence, the fusion protein having the substrate specificity of the sequence shown in SEQ ID NO:1.
25

It is also recognised that the peptide of the first aspect of the invention may be comprised in a polypeptide, so that the polypeptide has the substrate specificity of DPP8. The polypeptide may be useful, for example, for
30   altering the protease susceptibility of DPP8, when used in in vivo applications. An example of a polypeptide which may be useful in this regard, is albumin. Thus in another embodiment, the peptide of the first aspect is comprised in a polypeptide which has the substrate specificity of
35   DPP8.

As described above, the isolation and characterisation of DPP8 is necessary for identifying inhibitors of DPP8 catalytic activity, which may be useful for the treatment of disease. A method for identifying inhibitors of DPP8

5   catalytic activity, described herein, has identified that various inhibitors of DPPIV and serine proteases, zinc and mimetic peptides, Ala-Pro-Gly and Lys-Pro, but not inhibitors of metalloproteinases, aspartyl proteinases or cysteinyl proteinases, inhibit DPP8 catalytic activity.

10   Accordingly, in a fifth aspect, the invention provides a method of identifying a molecule capable of inhibiting cleavage of a substrate by DPP8, the method comprising the following steps:

      (a)   contacting DPP8 with the molecule;

15   (b)   contacting DPP8 of step (a) with a substrate capable of being cleaved by DPP8, in conditions sufficient for cleavage of the substrate by DPP8; and

      (c)   detecting substrate not cleaved by DPP8, to identify that the molecule is capable of inhibiting

20   cleavage of the substrate by DPP8.

It is recognised that although inhibitors of DPP8 may also inhibit DPPIV and other serine proteases, as described herein, the alignment of the DPP8 amino acid sequence with

25   most closely related molecules, (i.e. DPPIV), reveals that the DPP8 amino acid is distinctive, particularly at the regions controlling substrate specificity. Accordingly, it is expected that it will be possible to identify inhibitors which inhibit DPP8 catalytic activity

30   specifically, which do not inhibit catalytic activity of DPPIV-like gene family members, or other serine proteases. Thus, in a sixth aspect, the invention provides a method of identifying a molecule capable of inhibiting specifically, the cleavage of a substrate by DPP8, the

35   method comprising the following steps:

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(a)   contacting DPP8 and a further protease with the molecule;

(b)   contacting DPP8 and the further protease of step (a) with a substrate capable of being cleaved by DPP8

5   and the further protease, in conditions sufficient for cleavage of the substrate by DPP8 and the further protease; and

(c)   detecting substrate not cleaved by DPP8, but cleaved by the further protease, to identify that the

10   molecule is capable of inhibiting specifically, the cleavage of the substrate by DPP8.

In a seventh aspect, the invention provides a method of reducing or inhibiting the catalytic activity of DPP8, the

15   method comprising the step of contacting DPP8 with an inhibitor of DPP8 catalytic activity. As various inhibitors of DPPIV catalytic activity are shown herein to inhibit DPP8 catalytic activity, it is recognised that other inhibitors of DPPIV may be useful for inhibiting

20   DPP8 catalytic activity. Examples of inhibitors suitable for use in the seventh aspect are described in [21,32,33]. Other inhibitors useful for inhibiting DPP8 catalytic activity can be identified by the methods of the fifth or sixth aspects of the invention, which methods are

25   exemplified herein.

In one embodiment, the catalytic activity of DPP8 is reduced or inhibited in a mammal by administering the inhibitor of DPP8 catalytic activity to the mammal. It is

30   recognised that these inhibitors have been used to reduce or inhibit DPPIV catalytic activity *in vivo*, and therefore, may also be used for inhibiting DPP8 catalytic activity *in vivo*. Examples of inhibitors useful for this purpose are disclosed in the following [21,32-34].

35

Preferably, the catalytic activity of DPP8 in a mammal is reduced or inhibited in the mammal, for the purpose of

treating a disease in the mammal. Diseases which are
likely to be treated by an inhibitor of DPP8 catalytic
activity are those in which DPPIV-like gene family members
are associated [3,10,11,17,21,36], including for example,
5   neoplasia, type II diabetes, cirrhosis, autoimmunity,
graft rejection and HIV infection.

Preferably, the inhibitor for use in the seventh aspect of
the invention is one which inhibits the cleavage of a
10   peptide bond C-terminal adjacent to proline. As described
herein, examples of these inhibitors are 4-(2-
aminoethyl)benzenesulfonylfluoride, aprotinin,
benzamidine/HCl, Ala-Pro-Gly, H-Lys-Pro-OH HCl salt and
zinc ions, for example, zinc sulfate or zinc chloride.
15   More preferably, the inhibitor is one which specifically
inhibits DPP8 catalytic activity, and which does not
inhibit the catalytic activity of other serine proteases,
including, for example DPPIV or FAP.

20   In an eighth aspect, the invention provides a method of
cleaving a substrate which comprises contacting the
substrate with DPP8 in conditions sufficient for cleavage
of the substrate by DPP8, to cleave the substrate.
Examples of molecules which can be cleaved by the method
25   are H-Ala-Pro-pNA, H-Gly-Pro-pNA and H-Arg-Pro-pNA. The
conditions sufficient for cleaving the substrate are
described herein. Molecules which are cleaved by DPPIV
including RANTES, eotaxin, macrophage-derived chemokine,
stromal-cell-derived factor 1, glucagon and glucagon like
30   peptides 1 and 2, neuropeptide Y, substance P and
vasoactive peptide are also likely to be cleaved by DPP8
[11,12]. In one embodiment, the substrate is cleaved by
cleaving a peptide bond C-terminal adjacent to proline in
the substrate. The molecules cleaved by DPP8 may have
35   Ala, or Trp, Ser, Gly, Val or Leu in the P1 position, in
place of Pro [11,12].

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As described herein, DPP8 gene expression is upregulated in stimulated lymphocyte and lymphocytic cell lines which suggests that DPP8 may have a functional role in T cell costimulation and proliferation. It is recognised
5   therefore that measuring DPP8 gene expression is useful for detecting T cell activation. Thus in a ninth aspect, the invention provides a method of detecting an activated T cell, the method comprising the step of detecting the level of DPP8 gene expression in a T cell. In one
10   embodiment, the level of DPP8 gene expression is detected by measuring the amount of DPP8 mRNA in the cell, as described herein.

The inventors have characterised the sequence of a nucleic
15   acid molecule which encodes the amino acid sequence shown in SEQ ID NO:1. Thus in a tenth aspect, the invention provides a nucleic acid molecule which encodes the amino acid sequence shown in SEQ ID NO:1.

20   In an eleventh aspect, the invention provides a nucleic acid molecule which consists of the sequence shown in SEQ ID NO:2.

As described herein, at least three splice variants of
25   DPP8 RNA which have an open reading frame from 2.6 to 3.1 kb in length are observed. As a frame shift mutation or termination signal was not observed in the sequence of these splice variants, and as the coding sequence of two of the splice variants includes a sequence which encodes
30   the amino acid sequence associated with catalytic activity, it is recognised that some of the peptides encoded by the splice variants are likely to have the substrate specificity of DPP8. Thus in an embodiment, the nucleic acid molecule is a fragment of the sequence shown
35   in SEQ ID NO: 1 which is about 2.6 to 3.1 kb in length and which encodes a peptide which has the substrate

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specificity of the sequence shown in SEQ ID NO:1.
Preferably, the nucleic acid molecule has a sequence shown
in any one of SEQ ID NO.s: 4, 6 and 8.

5   In a twelfth aspect, the invention provides a nucleic acid
molecule which is capable of hybridising to a nucleic acid
molecule consisting of the sequence shown in SEQ ID NO:2
in stringent conditions, and which encodes a peptide which
has the substrate specificity of the sequence shown in SEQ
10   ID NO:1. As shown in the Northern blot analysis described
herein, DPP8 mRNA hybridises specifically to the sequence
shown in SEQ ID NO:2, after washing in 2XSSC/ 1.0%SDS at
37°C, or after washing in 0.1XSSC/0.1% SDS at 50°C.
"Stringent conditions" are conditions in which the nucleic
15   acid molecule is exposed to 2XSSC/ 1.0% SDS. Preferably,
the nucleic acid molecule is capable of hybridising to a
molecule consisting of the sequence shown in SEQ ID NO:2
in high stringent conditions. "High stringent conditions"
are conditions in which the nucleic acid molecule is
20   exposed to 0.1XSSC/ 0.1%SDS at 50°C.

As described herein, the inventors believe that the gene
which encodes DPP8 is located at band q22 on human
chromosome 15. The location of the DPP8 gene is
25   distinguished from genes encoding other prolyl
oligopeptidases, which are located on chromosome 2, at
bands 2q24.3 and 2q23, or chromosome 7. Thus in an
embodiment, the nucleic acid molecule is one capable of
hybridising to a gene which is located at band q22 on
30   human chromosome 15.

It is recognised that a nucleic acid molecule which
encodes the amino acid sequence shown in SEQ ID NO:1, or
which comprises has the sequence shown in SEQ ID NO:2,
35   could be made by producing the fragment of the sequence
which is translated, using standard techniques [30,31].

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Thus in an embodiment, the nucleic acid molecule does not contain 5' or 3' untranslated sequences.

In a thirteenth aspect, the invention provides a vector

5 which comprises a nucleic acid molecule of the tenth aspect of the invention. In one embodiment, the vector is capable of replication in a COS-7 cell, CHO cell or 293T cell, or E.coli. In another embodiment, the vector is selected from the group consisting of λTripleEx, pTripleEx,

10 pGEM-T Easy Vector, pSecTag2Hygro, pet15b, pEE14.HCMV.gs and pCDNA3.1/V5/His.

In a fourteenth aspect, the invention provides a cell which comprises a vector of the thirteenth aspect of the

15 invention. In one embodiment, the cell is an E.coli cell. Preferably, the E. coli is MC1061, DH5α, JM109, BL21DE3, pLysS. In another embodiment, the cell is a COS-7, COS-1, 293T or CHO cell.

20 In a fifteenth aspect, the invention provides a method for making a peptide of the first aspect of the invention comprising, maintaining a cell according to the fourteenth aspect of the invention in conditions sufficient for expression of the peptide by the cell. The conditions

25 sufficient for expression are described herein. In one embodiment, the method comprises the further step of isolating the peptide

In a sixteenth aspect, the invention provides a peptide

30 when produced by the method of the fifteenth aspect.

In a seventeenth aspect, the invention provides a composition comprising a peptide of the first aspect and a pharmaceutically acceptable carrier.

35

In an eighteenth aspect, the invention provides an antibody which is capable of binding a peptide according to the first aspect of the invention. The antibody can be prepared by immunising a subject with purified DPP8 or a
5   fragment thereof according to standard techniques [35]. As described herein, an antibody was prepared by immunising with transiently transfected DPP8^{+} cells. It is recognised that the antibody is useful for inhibiting activity of DPP8, or for detecting increased gene
10   expression of DPP8, for the purpose of identifying an activated T cell. In one embodiment, the antibody of the eighth aspect of the invention is produced by a hybridoma cell.

15   In a nineteenth aspect, the invention provides a hybridoma cell which secretes an antibody of the nineteenth aspect.

BRIEF DESCRIPTION OF THE FIGURES

20   Figure 1. Cloning strategy for isolating full-length DPP8 cDNA and the alternative splicing variants of DPP8 observed. Representation of three splice variants is shown including loss of serine recognition site by one splice variant (T8).

25

Figure 2. Nucleotide sequence and amino acid sequence of human DPP8. The nucleotide and predicted one letter code amino acid sequence are shown. This sequence shows no putative membrane spanning domain (deduced from
30   hydrophobicity plots) or potential N-linked glycosylation sites. The putative serine recognition site and aspartic acid and histidine which form the Ser-Asp-His catalytic triad are marked. Base pairs are numbered in the right margin.

35

Figure 3. Alignment of the deduced amino acid residue sequence of DPP8 with the *C. elegans* homolog of DPP8 and human DPPIV. Amino-acid residues are numbered in the right margin. Amino-acid residues identical in all three

5 proteins are boxed. Asterisks mark the putative catalytic triad residues and two glutamates of the β-propeller domain essential for DPPIV enzyme activity. The grey shading denotes the α/β hydrolase domain of these proteins. Filled triangles joined by lines indicate starts and ends of

10 alternatively spliced transcripts, stPBMCdy3-3-10 (solid lines), T8(dashed lines) and T21 (solid lines). The alignment was constructed using the PILEUP program in GCG.

Figure 4. Northern Blot analysis of DPP8 expression. Human

15 multiple tissue Northern blots (CLONTECH) containing 2 μg per lane of poly A$^+$ RNA were hybridized with a ^{32}P labeled DPP8 probe at 68°C and washed at high stringency. The autoradiograph was exposed for 1 day at -70°C with a BIOMAX MS screen. Molecular mass markers are indicated in base

20 pairs on the left side of each autoradiogram. Figure 4a. Master RNA (CLONTECH) blot of poly A$^+$ RNA was hybridized with a ^{32}P labelled DPP8 probe at 65°C and washed at high stringency. The autoradiograph was exposed for 3 days at -70°C with BIOMAX MS screen. DPP8 mRNA was detected in all

25 tissues examined.

Figure 5. Chromosomal localization of human DPP8. Metaphase showing FISH with the biotinylated DPP8 cDNA probe. Normal male chromosomes stained with DAPI.

30 Hybridization sites on chromosome 15 are indicated by an arrow.

Figure 6. Western blot analysis of transfected cell lines. Analysis of lysates of stable cell lines. DPP8 protein was

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seen in DPP8 /V5/His stable cell lines but not in DPP4 or vector-only stable cell lines. The electrophoretic mobility of the protein was not altered when samples were boiled. The band of greater mobility was probably a

5 breakdown product of intact DPP8.

Figure 7. DPP8 enzyme activity. (A) pH-dependence of DPP8 enzyme activity. (B) DPP8 and DPPIV enzyme kinetics. Means +/- SD of absorbance change per minute, multiplied

10 by 1000 are shown. Curve fitting assumed Michaelis-Menten kinetics.

Figure 8. RT-PCR analysis of DPP8 expression. PCR amplifications with primers specific for either a portion

15 of human DPP8 that contained no alternate splicing, Val416 to Gly 679 (top of each gel) or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (bottom of each gel. (A) Top gel, lanes 1-5 contain PCR products from unstimulated PBMC cDNA from five subjects. Bottom gel, lanes 6 to 11 contain PCR

20 products from OKT3-stimulated PBMC cDNA from six subjects. (B). PCR products are from cDNA from lymphocytic cell lines, liver or placenta as indicated. Negative control amplifications contained reaction mix, enzyme and no cDNA template. Each PCR was performed for 35 cycles. The PCR

25 products were electrophoresed on agarose gels and stained with ethidium bromide. The left lane of each gel contains PUC19 digested with *HaeIII* as size markers.

Figure 9. Northern blot analysis of murine DPP8

30 expression. A murine Northern blot containing 10 μg per lane of total RNA was hybridized with a ^{32}P-labeled human DPP8 probe at 60°C and washed at low stringency. Autoradiographic exposure was for 3 days at -70°C with a BIOMAX MS screen.

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DETAILED DESCRIPTION OF THE INVENTION

EXAMPLES

<u>General</u>

5   Restriction enzymes and other enzymes used in cloning were obtained from Boehringer Mannheim Roche. Standard molecular biology techniques were used [31] unless indicated otherwise.

10   An EST clone (GENBANK[TM] accession number AA417787) was obtained from American Type Culture Collection. The DNA insert of this clone was sequenced on both strands using automated sequencing at SUPAMAC (Sydney, Australia).

15   <u>Cell culture and RNA preparation</u>
     Human peripheral blood monocytes (PBMCs) were isolated by Ficoll-Hypaque density-gradient centrifugation (Pharmacia, Uppsala, Sweden) of blood obtained from healthy donors. The PBMCs were incubated in AIM-V medium (Life
20   Technologies, Gaithersburg, MD, USA) supplemented with 2 mM L-glutamine and were stimulated with either 1 μg.mL^{-1} phytohaemagglutinin (Wellcome) or 100ng.mL^{-1} OKT3 (Orthoclone, FL, USA) for 72 h. The human cell lines Jurkat, CCRF-CEM, Raji, Daudi and HepG2 were grown to
25   confluence in Dulbecco's modified Eagle's medium (Trace Biosciences, NSW, Australia) supplemented with 10% fetal bovine serum and 2mM L-glutamine.

     Liver and placental RNA were prepared from snap-frozen
30   human tissue as described previously [37]. However, RNA was prepared from PBMCs and cell lines using an RNAeasy kit (Qiagen, Germany).

     <u>Bioinformatics</u>
35   BLAST programs [38] and all multiple sequence alignments

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were performed through the Australian National Genomic
Information Service (ANGIS, Sydney, NSW, Australia).
PILEUP (GCG Version 8, Genetics Computer Group, Madison,
WI, USA) was used for multiple sequence alignments of
5   proteins.

A BLAST search was performed on the public expressed
sequence tag (EST) database using the complete human DPPIV
(GenBank™ accession number X60708) and FAP (accession
10   number U09278) nucleotide sequences as query sequences.
An EST clone (accession number AA417787) was obtained from
the American Type Culture Collection. The DNA insert of
this clone was sequenced on both strands using automated
sequencing at SUPAMAC (Sydney, NSW, Australia). Because
15   of its homology with DPPIV, this new gene was named
dipeptidyl peptidase 8 (DPP8).

DPP8 Cloning

ESTAA417787 was used to design forward (caa ata gaa att
20   gac gat cag gtg) and reverse (tct tga agg tag tgc aaa aga
tgc) DPP8 primers for polymerase chain reaction (PCR) from
ESTAA417787. The PCR conditions were as follows: 94°C for 5
min, followed by 35 cycles of 94°C for 1 minute, 55°C for
30 sec and 70°C for 1 min. This 484 bp PCR product was gel
25   purified, ^{32}P-α labelled using Megaprime Labeling Kit
(Amersham Pharmacia Biotec, UK, and hybridized to a Master
RNA blot (CLONTECH, Palo Alto, CA, USA) that contained
poly A⁺ from 50 adult and fetal tissues immobilized in dots
as per manufacturers' instructions. This Master RNA blot
30   was also probed with DPP4 for comparison of mRNA tissue
expression.

The forward and reverse DPP8 primers were used for PCR to
screen a human placental λ STRETCH PLUS library (CLONTECH,
35   Palo Alto, CA, USA) for the presence of DPP8 cDNA in the

library. The library was then screened by standard
molecular biology techniques [30,31]. After primary
screening, 23 clones were selected for secondary
screening, after which 22 remained positive. For the

5   tertiary screen the clones contained in λTripleEx were
converted into pTriplEx plasmids and transformed into
BM25.8 *E. coli* recipient bacteria. The plated bacteria
were screened and it was confirmed that all 22 clones were
positive. Two of these clones, T8 and T21 were selected

10   for further study.

5'RACE (Rapid amplification of cDNA ends)
A 5' RACE Version 2.0 kit (Gibco BRL, Life technologies)
was applied on activated T cell (ATC) and placental RNA as

15   prescribed in the kit instructions. The T8 DNA sequence
was used to design GSP 1 (TCC TTC CTT CAG CAT CAA TC) and
GSP2 (CTT AAA AGT GAC TTT AGG ATT TGC TGT ACC). 5' RACE
PCR products were cloned into pGEM-T Easy®Vector (Promega
Co., Madison, WI, USA) and sequenced by primer walking.

20

Confirmation of identity of RACE product
Reverse transcriptase PCR was carried out on ATC RNA using
DPP8-pr23 (GGA AGA AGA TGC CAG ATC AGC TGG) and DPP8-pr19r
(TCC GTG TAT CCT GTA TCA TAG AAG) to span across the

25   junction between the RACE product and the EST and library
clones. Two gel purified products ATCd3-2-1 (1603bp) and
ATC3-3-10 (1077bp) were cloned into pGEM-T Easy® (Promega
Co., Madison, WI, USA) and sequenced.

30   Subcloning of DPP8 cDNA into a pcDNA3.1/V5/His Expression
Vector
The ATC RACE product, the ATCd3-2-1 (1603bp) junction
fragment and the library clone T21 were joined together
and cloned into the expression vector pcDNA3.1/V5/His A

35   (Invitrogen, the Netherlands) to form a DPP8 cDNA of 3.1

kb with an open reading frame of 882 aa. The first
construct was made using three sequential cloning steps.
Firstly, a *Eco RV/Xba I* fragment of T21 (containing 3'
DPP8, stop codon and 3' untranslated region on DPP8 cDNA)
5   was ligated into the vector pcDNA3.1/V5/His A which had
been digested with *Eco RV/Xba I*. An *Eco RI/Eco RV* fragment
of ATCd3-2-1 was then added to this construct digested
with *Eco RI/Eco RV*. Finally the RACE product was cut with
Eco RI and cloned into the *Eco RI* site of the previous
10   construct to form the complete 3.1 kb DPP8 cDNA. This
construct pcDNA3.1-DPP8 expressed protein with no
detectable tag. In addition the stop codon in the DPP8
expression construct in pcDNA3.1/V5/His V5 was genetically
altered using PCR to create a C-terminal fusion with the
15   V5 and His tag contained in the vector. This construct was
named pcDNA3.1- DPP8/V5/His. All expression constructs
subcloned into pcDNA3.1/V5/His were verified by full
sequence analysis.

20   <u>DPP8 gene expression by Northern Blot</u>
Human multiple tissue Northern blots (CLONTECH) containing
2 ug of poly A$^+$ RNA were prehybridized in Express
Hybridization solution (CLONTECH) for 30 min at 68°C.
Both the DPP8 484 bp product and the 5' RACE ATC product
25   were radiolabeled using a Megaprime Labeling kit (Amersham
Pharmacia Biotech) and [^{32}P]dCTP (NEN Dupont).
Unincorporated label was removed using a NICK column
(Amersham Pharmacia Biotech) and the denatured probe was
incubated for 2 hrs at 68°C in Express Hybridization
30   solution. Washes were performed at high stringency and
blots exposed to BIOMAX MS film for overnight with a
BIOMAX MS screen at -70°C.

<u>DPP8 gene expression in mice by Northern Blot</u>

A Northern blot containing 10 ug of total liver RNA per lane was made using standard methods [31]. The RNA was derived from male and female mice of two strains, C57Bl6 and Balb/c. The Northern blot was prehybridized in Express
5 Hybridization solution (CLONTECH, Palo Alto, USA) for 1 hr at 60°C.   A 2.4kb human DPP8 cDNA (PCR product) was radiolabeled using the Megaprime Labeling kit (Amersham Pharmacia Biotech) and $[^{32}P]dCTP$ (NEN Dupont). Unincorporated label was removed using a NICK column
10 (Amersham Pharmacia Biotech) and the denatured probe was incubated with the blot overnight at 60°C in Express Hybridization solution. Washes were performed at low stringency (2 x SSC/0.05% SDS for 1 h at 37°C followed by 0.1x SSC/0.1% SDS for 30 min at 40°C) and blots exposed to
15 BIOMAX MS film for three days with a BIOMAX MS screen at - 70°C.

Expression of DPP8 in mouse liver using rtPCR

Mouse liver RNA was reverse transcribed using the
20 Superscript II enzyme kit (Gibco BRL, Gaithersburg, MD) as described previously [42]. The cDNA was diluted 1 in 4 and stored in aliquots at - 70°C. PCR using mouseDPP8-pr1F (atg att acc acc cag gaa gcg) as the forward primer and mouseDPP8-pr2R (atc tcc gac atc ttg aaa gtg acc) as the
25 reverse primer was used to detect mouse DPP8 mRNA.

      One ul of diluted cDNA was amplified in a 50 ul PCR reaction which contained: 0.2 mM dNTPs, 1 ul of 50 x Advantage 2 Polymerase Mix (Clontech), 1 X Advantage 2 PCR buffer (Clontech) and 100 ng of each primer. The PCR
30 involved an initial step of 95°C for 1 min to inactivate the TaqStart Antibody. This was followed by 35 cycles; denaturation at 95°C for 30 sec, 68°C for 1 min, followed by a final step of 68°C for 1 min. The amplified products were analysed by electrophoresis of 10 μl of PCR reaction
35 on a 3:1 Nusieve gel (FMC Bioproducts, Rockville, MD) plus

●   ●

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0.5 µg/ml ethidium bromide in TAE buffer (0.04M Tris acetate, 0.001 M EDTA, pH 8.0). The gel was then Southern Blotted using standard techniques [31]. The Southern blot was hybridized at 60°C for 2hr with the 2.4 kb human DPP8

5   cDNA probe prepared as described above. Washes were performed at low stringency (2 x SSC/0.05% SDS for 1 h at 37°C followed by 0.1x SSC/0.1% SDS for 40 min at 50°C). The blot was exposed to XAR5 Kodak film for 30 min at RT.

10   DPP8 expression by RT-PCR

Reverse transcriptase PCR was performed on human ATC RNA, human placental RNA and human liver RNA using TED primers DPP8/pr3 (GCA CTA CCT TCA AGA AAA CCT TGG) and DPP8/pr20R (TAT GGT ATT GCT GGG TCT CTC AGG) to give a 293 bp

15   product.

Transfection, Western blot, immunocytochemistry, cytochemistry and flow cytometry

Monkey kidney fibroblast (COS-7) cells (American Type

20   Culture Collection, CRL-1651) were grown and transfected as described previously [39]. For making stable cell lines, Geneticin (G418; Gibco-BRL) was added to the medium, beginning 24 h after transfection.. COS cell extracts were prepared by sonication followed by

25   differential centrifugation and neither boiled nor reduced before SDS/PAGE (10% gel) and transfer to nitrocellulose. as described previously [40,9]. The presence of DPP8 fused with the V5 epitope was detected using an anti-V5 mAb (Invitrogen). COS cell monolayers were fixed in cold

30   ethanol before staining with anti-V5 mAb [39,41,9]. Some monolayers were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 [35], then double-stained with wheat germ agglutinin to label Golgi apparatus and with goat anti-mouse IgG to label DPP8,

35   conjugated to Alexa Fluor 488 and Alexa Fluor 594,

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respectively (Molecular Probes, Eugene, OR, USA). Flow
cytometry and confocal scanning microscopy using a Leica
TCS-NT confocal microscope have been described previously
[39,9].

5

Purification of recombinant DPP8/V5/His and DPPIV/V5/His

Cells (1×10^7) expressing each protein were sonicated in
native buffer (50mm sodium phosphate, 300 mm NaCl), then
10   treated with 700 U DNAse for 20 min at room temperature.
DPPIV is expressed at the cell surface, so 1% Triton X-100
was used to solubilize DPPIV/V5/His. Insoluble material
was removed by centrifugation. The supernatant was
incubated with 1 mL Talon® Metal Affinity Resin (Clontech)
15   following the manufacturer's instructions for a
batch/gravity flow procedure. The resin was washed with
50 mm sodium phosphate, containing 300 mm NaCl and 5 mm
imidazole, and proteins were eluted using the same buffer
containing 150 mm imidazole. Enzyme activity was used to
20   monitor eluted fractions.

Enzyme assays

Enzyme assays were performed as described previously [1].
Either clarified cell extract from 1×10^4 sonicated COS-7
25   cells or purified protein derived from 1×10^5 cells was
incubated with substrate in 70μL phosphate buffer, pH 7.4,
for 30 min at 37°C, except where otherwise indicated. The
specific DPPIV substrates, Gly-Pro-toluenesulfonate, H-
Gly-Pro-p-nitroanilide (NA)/HCl (Sigma, St Louis, MO, USA)
30   and Gly-Pro-7-amino-4-trifluromethylcoumarin (Calbiochem,
San Diego, CA, USA) were tested. Other substrates tested
were H-Ala-Pro-pNA/HCl, H-Arg-Pro-pNA acetate salt, H-Lys-
Ala-pNA.2HCl, H-Asp-Pro-pNA, H-Ala-Ala-pNA/HCl, H-Ala-Ala-
Pro-pNA/HCl, H-Ala-Ala-Phe-pNA, succinyl-Ala-Pro-pNA, H-
35   Ala-Phe-Pro-pNA and Z-Ala-Pro-p-NA from Bachem

(Switzerland).   H-Ala-Pro-4-methoxyβNA/HCl, Z-Lys-Pro-4-
methoxyβNAformate salt, H-Lys-Pro-4-methoxyβNA/HCl, Z-Ala-
Pro-4-methoxyβNA, H-Gly-Pro-βNA and H-His-Ser-4-
methoxyβNAacetate salt (Bachem) were tested for their

5   ability to stain unfixed transfected cells.

All inhibitors were (see Table 2) incubated with each
purified enzyme in phosphate buffer, pH 7.4, for 15 min
before the addition of substrate.   After the addition of
1mM H-Ala-Pro-pNA substrate for purified DPP8 and 1 mM H-

10   Gly-Pro-pNA substrate for purified DPPIV, samples were
incubated for 60 min at 37°C.   All enzyme assays were
performed in triplicate.

Chromosomal localization of DPP8 by Fluorescence in situ

15   Hybridization (FISH) analysis

DPP8 was localized using two different probes, the DPP8
EST and the T8 clone. The probes were nick-translated with
biotin-C^{14}-dATP and hybridized in situ at a final
concentration of 10ng/ul to metaphases from two normal

20   males. The FISH method was modified from that previously
described [37] in that chromosomes were stained before
analysis with both propidium iodide (as counterstain) and
DAPI (for chromosomal identification). Images of metaphase
preparations were captured by a cooled CCD camera using

25   the Cyto Vision Ultra image collection and enhancement
system (Applied Imaging International Ltd).   FISH signals
and the DAPI banding pattern were merged for figure
preparation.

30   Expression of DPP8 in human lymphocytes and cell lines

RNA (1µg) was reverse-transcribed using the Superscript II
enzyme kit (Gibco-BRL) as described previously [42].   PCR
using DPP8-pr18 (CTGTGACGCCACTAATTATCTATG) as the forward
primer and DPP8-pr26R (CCTAGAGAGGCTAGGGTATTCAAG) as the

- 24 -

reverse primer was used to detect full-length DPP8 mRNA.
The glyceraldehyde-3-phosphate dehydrogenase (G3PDH)
control primer set was G3PDH for (ACCACAGTCCATGCCATCAC)
and G3PDHrev (TCCACCACCCTGTTGCTGTA) to give a 470-bp

5   product.

cDNA (diluted 1 : 4; 1μg) was amplified in a 25-μL PCR
mixture which contained: 0.2 mM dNTPs, 0.125 unit Amplitaq
Gold enzyme (Perkin-Elmer), 1 x buffer II (Perkin-Elmer),

10   1.5 mM $MgCl_2$ and 100ng mL^{-1} each primer. The 35-cycle PCR
was performed as follows: denaturation at 94°C for 1 min,
primer annealing at 55°C for 30 s, and an extension step at
72°C for 1 min. The amplified products were analyzed by
electrophoresis of 15μL PCR mixture on a 3 : 1 Nusieve gel

15   (FMC Bioproducts, Rockville, MD, USA) plus 0.5 μg mL^{-1}
ethidium bromide in Tris/acetate/EDTA buffer (0.04 M
Tris/acetate, 0.001 M EDTA, pH 8.0).

Anti-peptide antibody

20   Methods followed are described in Current Protocols in
Immunology [35]. Two peptides were chosen using the
software MacVector to predict antigenicity. The two
peptides were custom synthesized (Auspep, Melbourne) and
conjugated to diptheria toxin (Auspep, Melbourne).

25   Rabbits were immunized with both peptides and serum
collected at time zero and after each injection (IMVS,
Adelaide.

The two peptides used were:

30

PEPTIDE Name: TEDDA-N
SEQUENCE: CTGYTERYMGHPDQNEQG-NH2

This is amino acids 773 to 789, plus a Cys at the N-

terminus.

PEPTIDE Name: TEDDR-C
SEQUENCE: GKPYDLQIYPQERHSC-NH2

This is amino acids 836 to 850, plus a Cys at the C-terminus.

These sequences were taken from the C-terminal portion of DPP8.

Monoclonal antibody to DPP8

Standard methods were used for antibody production [35]. Mice were immunized with 2 x10^7 live COS-7 (African Green Monkey Kidney) cells that had been transiently transfected with the DPP8 cDNA in the pcDNA3 vector. The final immunisation was with CHO (Chinese Hamster Ovary) cells stably transfected with DPP8 cDNA in the pEE14 vector. Spleen cells were fused with a standard fusion partner, X63Ag8 myeloma cells. Hybridoma culture supernatants were tested by immunoperoxidase histochemistry on monolayers of the DPP8-transfected CHO cell line, using untransfected CHO cells as the negative control. Hybridomas that produced antibody activity were cloned.

RESULTS

Molecular cloning and sequence analysis of DPP8
The insert in ATCC EST AA417787 was 795 bp in length, containing 527 bp of coding sequence, a TAA stop codon and 258 bp of 3' noncoding sequence (Figure 1).

The hybridization of the Master RNA blot revealed that the gene comprising ESTAA417787 has ubiquitous tissue expression, with high levels of expression in testis and placenta. Based on this expression pattern, a placental

cDNA library was screened with a 484 bp PCR product
produced by the forward and reverse DPP8 primers.
Sequence homology analysis revealed that only 2 of 23
clones contained 5' sequence additional to the sequence of
5   ESTAA417787. These cDNA clones were designated T8 and
T21, and were 1669 bp and 1197 bp respectively (Figure 1).
In addition, comparison of these sequences to ESTAA417787
revealed that T8 cDNA lacked a 153 bp (51aa) region that
was present in T21 cDNA and ESTAA417787. This deletion
10   would result in the loss of the catalytic serine (GWSYGG)
in T8 cDNA. Many of the other clones characterized
appeared to contain unrelated sequence which are probably
intronic sequences as a result of incomplete splicing.

15   The 5' RACE technique was utilized on both ATC RNA and
placental RNA to obtain the 5' end of the DPP8 gene. The
RACE product obtained from activated T cell RNA was 0.2 kb
larger than that from placental RNA but otherwise
identical (Figure 1). The first methionine within a Kozak
20   sequence was found 214 bp from the 5' end of the activated
T cell RACE product. This 5' 211bp region was 70.5 % GC
rich and contained a number of potential promoter and
enhancer elements (Sp1, Ap1 and ETF sites) and so was
deduced to be the 5' flanking region of the DPP8 gene. In
25   order to confirm the identity of the 5' RACE product as
the 5' end of DPP8, RT-PCR was carried out to span across
the junction between the RACE product and T8 cDNA library
clone. The RT-PCR on ATC RNA produced two clones ATCd3-2-
1 and ATC3-3-10 (Figure 1). Compared to T8 and T21, both
30   clones had an additional insert region of 144bp (48 aa)
immediately adjacent to the splice site of T8. Sequence
homology analysis of this additional insert region found a
homologous region in both the C. elegans homologue and
DPP4. This clearly showed that T8 and T21 library clones
35   represented splice variants of DPP8. The smaller clone

ATCd3-3-10 was also found to represent another splice
variant of DPP8 as it contained a 516 bp deletion at the
5' end which would result in a deletion of 175 aa.

5   A full-length DPP8 clone was created using the larger RACE
     product, ATC3-2-1 and the T21 library clone. This
     generated a putative DPP8 cDNA of 3.1 kb (including 5' and
     3' untranslated regions) with an open reading frame of 882
     aa for further sequence analysis and examining DPP8
10   function. This 882 putative DPP8 protein contained no N-
     linked glycosylation sites and Kyte-Doolittle
     hydrophobicity analyses revealed it lacked a transmembrane
     domain, unlike DPP4, FAP and DPP6. Thus it is likely that
     DPP8 is a cytoplasmic protein (Figure 2). The predicted
15   DPP8 protein shared 51 % amino acid similarity and 27 %
     amino acid identity with human DPP4; the C termini of
     these proteins exhibited the most homology (Figure 3).

     <u>Tissue distribution of DPP8 as determined by Master RNA</u>
20   <u>and Northern Blot</u>
     A master RNA blot was probed with a 484 nt PCR product
     produced by the forward and reverse DDP8 primers as
     mentioned previously. The mRNA tissue expression of DPP8
     was ubiquitous in all human adult and fetal tissues. A
25   similar ubiquitous expression pattern was observed using
     DPP4 cDNA as a probe (data not shown). However, by visual
     assessment the greatest levels of expression using each
     gene specific probe were in different tissues. The most
     intense signals using the DPP8 probe were in testis
30   followed by placenta whereas the most intense signals
     using the DPP4 probe were in salivary gland and prostate
     gland followed by placenta (data not shown). The probes
     did not bind any of the negative controls on the blot.

35   Northern blot analysis was performed on mRNA derived from

- 28 -

different human tissues (Figure 4). Two DPP8 specific probes indicated the presence of transcripts in all tissues examined. A transcript approximately 3.0 kb in size consistent with the approximate expected size of DPP8

5 message was detected only in the testis. However, two transcripts of 8.0 and 5.0 kb respectively were present in testis, spleen, peripheral blood leukocytes and ovary at high levels; in prostrate, small intestine, and colonic mucosa at moderate levels; and in the thymus at lower

10 levels. The Multiple tissue Northern blot was also probed with radiolabeled human β-actin probe and a common 2.0 kb transcript was seen in all tissues (Figure 4).

Expression of DPP8 in mice determined by Northern Blot and

15 rtPCR.

The human DPP8 cDNA sequence cross-hybridized with murine derived liver RNA. The Northern blot containing total RNA from mouse liver hybridized to a human DPP8 probe, showing that DPP8 mRNA is expressed in mouse liver (Figure 9A).

20 Two mRNA transcripts of murine DPP8 were present. This is a similar pattern to that observed for human DPP8. These transcripts probably represent different length 5' and 3' untranslated regions of the murine DPP8 gene. The presence of DPP8 mRNA in the mouse liver was also demonstrated

25 using rt-PCR. The primers tested generated a 537bp PCR product. A Southern blot of this product confirmed that the murine DPP8 cross hybridizes with human DPP8 (Figure 9B).

30 Expression and functional activity of DPP8

To assess the function of DPP8 protein, the full length DPP8 cDNA of 3.1 kb was cloned into the *Xba I* site of pcDNA3.1A/V5/His expression vector to produce two constructs. The first construct, pcDNA3.1-DPP8, expressed

35 DPP8 protein on its own whilst the second construct,

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pcDNA3.1-DPP8/V5/His expressed a protein with the V5
epitope and His tag fused to the C-terminus of DPP8 to
facilitate analysis of protein expression. Mammalian
expression constructs were stably transfected into COS-7
5   cells and cellular sonicates prepared. Consistent with the
molecular weight predicted from the amino acid sequence a
100 kDa monomer was detected by Western blotting of stable
DPP8/V5/His expressing cells (Figure 6). DPP8/V5/His
protein was detected in the cytoplasmic compartment but
10   not on the surface of ethanol fixed stable DPP8/V5/His
expressing COS cells, using the anti-V5 mAb.

DPP8 is a dipeptidyl peptidase

Sequence homology between DPPIV and DPP8 suggested
15   functional similarities, so cell lysates of DPP8-
transfected cells were examined for proline-specific
peptidase activity. DPPIV expressed in COS-7 cells with
or without the V5/His tag were positive controls, and
negative controls included vector-only transfected COS07
20   cells. Extracts of DPP8-transfected COS-7 cells
hydrolyzed H-Ala-Pro-pNA and H-Arg-Pro-pNA but not H-Gly-
Pro-pNA, H-Gly-Arg-pNA, H-Gly-Pro-toluenesulfonate or H-
Gly-Pro-7-amino-4-trifluoromethylcoumarin above the levels
exhibited by untransfected COS-7 cells (data not shown).
25   The pH optimum of DPP8 enzyme activity was 7.4 (Fig. 5A),
similar to the pH 7.8 optimum DPPIV enzyme activity
[43,44]. DPP8 exhibited little activity below pH 6.3,
suggesting that it is not an enzyme of the
lysosome/endosome compartment. Of all the substrates
30   tested on cell monolayers, only Ala-Pro-4MβNA/HCl stained
DPP8-transfected COS cells and CHO cells (data not shown).

Both purified recombinant DPP8/V5/His and purified
recombinant DPPIV/V5/His hydrolyzed H-Ala-Pro-pNA, G-Gly-
35   Pro-pNA and H-Arg-Pro-pNA. Transfection with DPP8

possibly causes increased dipeptidase, tripeptidase and endopeptidase activities, similar to an effect of DPPIV transfection of melanoma cells [18]. Indeed, our results showed that DPP8 transfected COS-7 cells, but not purified

5 recombinant DPP8, exhibited tripeptidyl peptidase activity using the substrate H-Ala-Ala-Pro-pNA and endopeptidase activity using the substrate Z-Ala-Pro-pNA (data not shown). This was investigated further, and neither of the tripeptidyl peptidase substrates H-Ala-Ala-Phe-pNA or H-

10 Ala-Phe-Pro-pNA [45] nor the prolyl endopeptidase substrates Z-Ala-Pro-pNA or succinyl-Ala-Pro-pNA were cleaved by purified DPP8. Our data clearly demonstrate that DPP8 is a dipeptidyl peptidase and lacks tripeptidyl peptidase or endopeptidase activities.

15

The nature of the catalytic mechanism of DPP8 was further investigated using various inhibitors. DPP8 enzyme activity was significantly inhibited by serine proteinase inhibitors and was insensitive to inhibitors of

20 metalloproteinases, aspartyl proteinases and cysteine proteinases. DPP8 enzyme activity was significantly inhibited by zinc, which completely inhibits DPPIV enzyme activity [46]. The peptides Ala-Pro-Gly and Lys-Pro mimic DPP8 substrates and probably competitively inhibited DPP8.

25

Chromosomal localization of DPP8

Two probes were used for FISH analysis, ESTAA417787 and the T8 clone from the placental library. Seventeen metaphases from the first normal male were examined for

30 fluorescent signal. All of these metaphases showed signal on one or both chromatids of 15 at band q22 (Figure 5). There were a total of 2 non-specific background dots observed in these metaphases. A similar result was obtained from the hybridization of the probe to 15

35 metaphases from the second normal male (data not shown).

Analysis of *DPP8* gene expression by RT-PCR

DPPIV is expressed by most lymphocytes and lymphocytic cell lines but upregulated on activated lymphocytes [47, 41, 48, 49]. The various splice variants of DPP8 might not encode functional protein, so the PCR was designed to detect only mRNA that contained full-length sequence (Fig. 1). At 35 cycles, amplification product of the expected size (783 bp) was readily observed in OKT3-stimulated PBMCs (six of six subjects; Fig 8) but not in unstimulated PBMCs from most subjects (four of five, Fig. 8A), suggesting that more DPP8 mRNA is expressed in activated T cells than in unstimulated PBMCs. Similar RT-PCR data were obtained from PBMCs stimulated with phytohaemagglutinin (data not shown). In addition, DPP8 mRNA was expressed in all B and T cell lines examined and in both liver and placenta(Fig. 8B).

Anti-peptide antibody

The sera of two rabbits were tested by ELISA in peptide-coated wells. Both sera bound both peptides whereas the pre-immunisation serum samples did not exhibit specific binding. Western blots on extracts of cell lines, cell lines transfected with DPP8 cDNA and activated human lymphocytes showed that a rabbit antiserum to the two DPP8 peptides binds a 100kDa band which is the size of DPP8. (Data not shown).

Table 1. K_m and V_{max} values for DPP8 and DPPIV

	K_m (mM)		V_{max} (ΔA min^{-1} x 1000)	
	DPPIV	DPP8	DPPIV	DPP8
H-Ala-Pro-pNA	0.374 ± 0.134	0.991 ± 0.171	9.6 ± 1.0	12.4 ± 0.9
H-Gly-Pro-pNA	0.347 ± 0.088	0.467 ± 0.064	7.2 ± 0.49	3.5 ± 0.14

5

Table 2. Inhibition of the peptidase activity of DPP8 in
10 **comparison with DPPIV.** Common proteinase inhibitors of
various enzyme types were incubated with the purified
peptidases before assay with the substrates H-Ala-Pro-pNA
on DPP8 or H-Gly-Pro-pNA on DPPIV. AEBSF, 4-(2-
aminoethyl)benzenesulfonylfluoride.

15

		Residual activity (% of control)	
Type of inhibitor	Concentration	DPP8	DPPIV
None		100	100
Serine proteinase			
AEBSF	4 mM	40	52
Aprotinin	4 µg mL^{-1}	47	81
Benzamidine/HCl	10 mM	82	89
Peptides			
Gly-Gly-Gly	10 mM	99	106
Ala-Pro-Gly	10 mM	51	67
H-Lys-Pro-OH HCl salt	4 mM	63	45
Zinc sulphate	1 mM	25	0
Metalloproteinase			
EDTA	2 mM	115	99
Aspartate(acidic) proteinase			
Pepstatin	2 µg mL^{-1}	107	110
Leupeptin	0.1 mM	93	104
Cysteine(thiol) proteinase			
Iodoacetamide	2 mM	100	115
Dithiothreitol	2 mM	108	109

●

●

Discussion

We describe the cloning, recombinant expression, biochemistry and tissue expression of a novel human DPPIV-
5   related postproline peptidase that we have named DPP8. DPP8 exhibited dipeptidyl aminopeptidase but not tripeptidyl peptidase or endopeptidase activity. Like DPPIV, DPP8 was found to exhibit significant mRNA expression in activated T cells. Clear indications that
10   DPP8 is a monomeric, nonglycosylated, soluble, cytoplasmic protein, which are characteristics of PEP but not of DPPIV, FAP or DPP6, were provided by our sequence and localisation data. DPP8 enzyme activity had a neutral pH optimum, suggesting that it is not active in the acidic
15   lysosome/endosome compartment.

By homology with DPPIV, DPP8 is a member of the DPPIV-like gene family, a member of the prolyl oligopeptidase family S9b, and a member of the enzyme clan SC. The residues in
20   DPP8 that potentially form the charge-relay system are Ser739, Asp817 and His849 (Fig.2). The dipeptidyl peptidase activity of DPP8 and the absence of detectable tripeptidyl peptidase or endopeptidase activities by purified DPP8 further support its placement in the S9b
25   family. Furthermore, the DPP8 substrate specificity was indistinguishable from that of the structurally related peptidases DPPIV and FAP.

The role of DPPIV in human lymphocytes has been studied in
30   detail using enzyme inhibitors [49, 50-54]. DPPIV-
specific inhibitors suppress both DNA synthesis and cytokine production in vitro [48, 49, 52]. In addition, DPPIV-specific inhibitors decrease phorbol myristate acetate-induced tyrosine phosphorylation in human
35   lymphocytes, further suggesting a role for DPPIV enzyme

- 34 -

activity in lymphocyte activation [54]. *In vivo*, inhibitors of DPPIV suppress arthritis [20] and prolong cardiac allograft survival in animal models [55]. The ability of DPP8 to cleave DPPIV substrates indicates that
5   DPPIV inhibitors may also inhibit DPP8 and that inhibitor studies may require further interpretation. Indeed, DPP8 may be responsible for some of the physiological functions that have been assigned to DPPIV.

10 FAP and DPPIV are integral membrane glycoproteins and require dimerization for catalytic activity [9, 56, 57]. In contrast, DPP8 and PEP are non-glycosylated cytosolic proteins that are catalytically active as monomers [58] and cleave Pro-Xaa bonds [43,59]. However, the substrate
15 specificity of PEP is distinct from DPP8. PEP is an endopeptidase that does not cleave if a free α-amine lies N-terminal to the proline (e.g. it does not cleave H-Ala-Pro). Recently we have proposed that the tertiary structure of DPPIV is similar to that of PEP in having a
20 seven-blade β-propeller domain and an α/β-hydrolase domain [3, 39, 1]. The significant sequence identity between DPP8 and DPPIV indicates that the tertiary structures of DPP8 and DPPIV are similar. However, DPP8 contains 110 amino acids more than DPPIV, so it could have an
25 additional element of tertiary structure such as an eighth propeller blade.

The ancestral relationships between DPP8, DPPIV and FAP are reflected in their chromosomal localization. While
30 DPPIV and FAP have both been localized to the long arm of chromosome 2, 2q24.3 [60] and 2q23 [61] respectively, DPP8 was localized to 15q22. The related genes DPP6 and PEP have been localized to chromosome 7 [62] and 6q22 respectively [63].

35

- 35 -

Two human disease loci have been mapped to 15q22. These
loci are an autosomal recessive deafness locus [64] and a
form of Bardet-Biedl syndrome, type 4 [65]. Two of the
clinical manifestations of Bardet-Biedl syndrome are
5   obesity and diabetes. Attractin [66] and DPPIV have roles
in obesity [67] and diabetes [22, 68, 15] respectively and
as their substrate specificities overlap with that of
DPP8, it is possible that DPP8 may be involved in Bardet-
Biedl syndrome.

10

DPPIV is expressed on the surface of T cells and is a
costimulatory molecule called CD26 [3]. CD26-negative cell
lines have residual DPPIV enzyme activity and PBMC have
non-DPPIV derived activity against Ala-Pro substrates
15   [69], indicating the existence of other peptidase(s) with
DPPIV-like activity. DPPIV-β exhibits a peptidase activity
similar to DPPIV but is a 70-80 kDa cell surface
glycoprotein [70] and is therefore distinct from DPP8.

20   The biological significance of the three splice variants
of DPP8 that we discovered is unknown. None of these
splice variants result in a frame shift or premature
protein termination (Fig. 1). Two of the splice variants
contain all the predicted catalytic triad residues and
25   thus potentially produce proteins with peptidase activity.
Alternate splice forms of FAP mRNA have also been observed
[71, 72]. It is possible that expression of splice
variants may be used to regulate the levels of active
protein. DPP8 Northern blots revealed a number of
30   differently sized transcripts. The predicted sizes of
splice variants of DPP8 ranged from 2.6 to 3.1 kb whereas
the large transcripts seen in most tissues examined in the
Northern blots were 8.5 kb and 5.0 kb respectively.
Similarly, two other members of the DPPIV-like gene
35   family, DPPIV and DPP6, exhibit mRNA transcripts in

Northern blots that are much larger than the cDNA size
[60, 61]. We propose that the major transcripts for DPP8
mRNA and its splice variants lie within the 5 kb band
while the 8.5 kb transcript(s) may contain additional 5'
5   and 3' untranslated sequences. DPP8 appears to be like
DPPIV in having a ubiquitous mRNA expression pattern by
Northern analysis while being upregulated in activated T
cells. The similarities between DPP8 and DPPIV suggest
that DPP8 may, like DPPIV, play a role in T cell
10   costimulation and proliferation. The development of DPP8
specific antibodies or inhibitors will facilitate work in
this area.

In summary, we have identified and characterized a novel
15   human dipeptidyl aminopeptidase DPP8 with structural and
functional similarities to DPPIV and FAP. With many
diverse biological roles suggested for DPPIV, particularly
in the immune system, and the roles of FAP in tumor growth
and liver disease, it will be interesting to investigate
20   the roles of this new member of the DPPIV-like gene family
in these systems. Further work in understanding this novel
protein and the elucidation of inhibitors and
physiological substrates will help identify the specific
functions of individual members of this gene family.

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gagtggaggc ggcgcagcat gaagcggcgc aggcccgctc catagcgcac gtcgggacgg
    180

ttcggacggt accggaggca aggaaaatcc aacatgcac caccattca aacaggtgc
    240

ctgggtgttg agatatttga aactgcggac tgtgaggaga atattgaatc acaggatcgg
    300

cctaaattgg agcctttta tgttgagcgg tattcctgga gtcagcttaa aaagctgctt
    360

gccgatacca gaaaatatca tggctacatg atggctaagg caccacatga tttcatgttt
    420

1.ST25.txt

```
gtgaagagga atgatccaga tggacctcat tcagacagaa tctattacct tgccatgtct
480

ggtgagaaca gagaaaatac actgttttat tctgaaattc ccaaaactat caatagagca
540

gcagtcttaa tgctctcttg gaagcctctt ttggatcttt ttcaggcaac actggactat
600

ggaatgtatt ctcgagaaga agaactatta agagaaagaa aacgcattgg aacagtcgga
660

attgcttctt acgattatca ccaaggaagt ggaacatttc tgtttcaagc cggtagtgga
720

atttatcacg taaaagatgg agggccacaa ggatttacgc aacaaccttt aaggcccaat
780

ctagtggaaa ctagttgtcc caacatacgg atggatccaa aattatgccc cgctgatcca
840

gactggattg cttttataca tagcaacgat atttggatat ctaacatcgt aaccagagaa
900

gaaaggagac tcacttatgt gcacaatgag ctagccaaca tggaagaaga tgccagatca
960

gctggagtcg ctacctttgt tctccaagaa gaatttgata gatattctgg ctattggtgg
1020

tgtccaaaag ctgaaacaac tcccagtggt ggtaaaattc ttagaattct atatgaagaa
1080

aatgatgaat ctgaggtgga aattattcat gttacatccc ctatgttgga aacaaggagg
1140

gcagattcat tccgttatcc taaaacaggt acagcaaatc ctaaagtcac ttttaagatg
1200

tcagaaataa tgattgatgc tgaaggaagg atcatagatg tcatagataa ggaactaatt
1260

caaccttttg agattctatt tgaaggagtt gaatatattg ccagagctgg atggactcct
1320

gagggaaaat atgcttggtc catcctacta gatcgctccc agactcgcct acagatagtg
1380

ttgatctcac ctgaattatt tatcccagta gaagatgatg ttatggaaag gcagagactc
1440

attgagtcag tgcctgattc tgtgacgcca ctaattatct atgaagaaac aacagacatc
1500

tggataaaata tccatgacat ctttcatgtt tttccccaaa gtcacgaaga ggaaattgag
```

1.ST25.txt

```
1560
tttatttttg cctctgaatg caaaacaggt ttccgtcatt tatacaaaat tacatctatt
1620
ttaaaggaaa gcaaatataa acgatccagt ggtgggctgc ctgctccaag tgatttcaag
1680
tgtcctatca aagaggagat agcaattacc agtggtgaat gggaagttct tggccggcat
1740
ggatctaata tccaagttga tgaagtcaga aggctggtat attttgaagg caccaaagac
1800
tcccctttag agcatcacct gtacgtagtc agttacgtaa atcctggaga ggtgacaagg
1860
ctgactgacc gtggctactc acattcttgc tgcatcagtc agcactgtga cttctttata
1920
agtaagtata gtaaccagaa gaatccacac tgtgtgtccc tttacaagct atcaagtcct
1980
gaagatgacc caacttgcaa aacaaaggaa ttttgggcca ccattttgga ttcagcaggt
2040
cctcttcctg actatactcc tccagaaatt ttctcttttg aaagtactac tggatttaca
2100
ttgtatggga tgctctacaa gcctcatgat ctacagcctg gaaagaaata tcctactgtg
2160
ctgttcatat atggtggtcc tcaggtgcag ttggtgaata atcggtttaa aggagtcaag
2220
tatttccgct tgaataccct agcctctcta ggttatgtgg ttgtagtgat agacaacagg
2280
ggatcctgtc accgagggct taaatttgaa ggcgccttta aatataaaat gggtcaaata
2340
gaaattgacg atcaggtgga aggactccaa tatctagctt ctcgatatga tttcattgac
2400
ttagatcgtg tgggcatcca cggctggtcc tatggaggat acctctccct gatggcatta
2460
atgcagaggt cagatatctt cagggttgct attgctgggg ccccagtcac tctgtggatc
2520
ttctatgata caggatacac ggaacgttat atgggtcacc ctgaccagaa tgaacagggc
2580
tattacttag gatctgtggc catgcaagca gaaaagttcc cctctgaacc aaatcgttta
2640
```

1.ST25.txt

```
ctgctcttac atggtttcct ggatgagaat gtccattttg cacataccag tatattactg
2700

agttttttag tgagggctgg aaagccatat gattacagat ctatcctca ggagagacac
2760

agcataagag ttcctgaatc gggagaacat tatgaactgc atcttttgca ctaccttcaa
2820

gaaaaccttg gatcacgtat tgctgctcta aaagtgatat aattttgacc tgtgtagaac
2880

tctctggtat acactggcta tttaaccaaa tgaggaggtt taatcaacag aaaacacaga
2940

attgatcatc acattttgat acctgccatg taacatctac tcctgaaaat aaatgtggtg
3000

ccatgcaggg gtctacggtt tgtggtagta atctaatacc ttaaccccac atgctcaaaa
3060

tcaaatgata catattcctg agagacccag caataccata agaattacta aaaaaaaaaa
3120
```

```
<210>  3
<211>  310
<212>  PRT
<213>  Homo sapiens

<400>  3
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```
Phe Glu Gly Thr Lys Asp Ser Pro Leu Glu His His Leu Tyr Val Val
1               5                   10                  15

Ser Tyr Val Asn Pro Gly Glu Val Thr Arg Leu Thr Asp Arg Gly Tyr
                20                  25                  30

Ser His Ser Cys Cys Ile Ser Gln His Cys Asp Phe Phe Ile Ser Lys
            35                  40                  45

Tyr Ser Asn Gln Lys Asn Pro His Cys Val Ser Leu Tyr Lys Leu Ser
        50                  55                  60

Ser Pro Glu Asp Asp Pro Thr Cys Lys Thr Lys Gly Phe Trp Ala Thr
65                  70                  75                  80

Ile Leu Asp Ser Ala Gly Pro Leu Pro Asp Tyr Thr Pro Pro Glu Ile
                85                  90                  95

Phe Ser Phe Glu Ser Thr Thr Gly Phe Thr Leu Tyr Gly Met Leu Tyr
                100                 105                 110

Lys Pro His Asp Leu Gln Pro Gly Lys Lys Tyr Pro Thr Val Leu Phe
```

1.ST25.txt

```
              115                      120                         125
Ile Tyr Gly Gly Pro Gln Gly Gln Ile Glu Ile Asp Asp Gln Val Glu
         130                 135                 140

Gly Leu Gln Tyr Leu Ala Ser Arg Tyr Asp Phe Ile Asp Leu Asp Arg
145                 150                 155                 160

Val Gly Ile His Gly Trp Ser Tyr Gly Gly Tyr Leu Ser Leu Met Ala
                 165                 170                 175

Leu Met Gln Arg Ser Asp Ile Phe Arg Val Ala Ile Ala Gly Ala Pro
             180                 185                 190

Val Thr Leu Trp Ile Phe Tyr Asp Thr Gly Tyr Thr Glu Arg Tyr Met
             195                 200                 205

Gly His Pro Asp Gln Asn Glu Gln Gly Tyr Tyr Leu Gly Ser Val Ala
     210                 215                 220

Met Gln Ala Glu Lys Phe Pro Ser Glu Pro Asn Arg Leu Leu Leu Leu
225                 230                 235                 240

His Gly Phe Leu Asp Glu Asn Val His Phe Ala His Thr Ser Ile Leu
             245                 250                 255

Leu Ser Phe Leu Val Arg Ala Gly Lys Pro Tyr Asp Leu Gln Ile Tyr
             260                 265                 270

Pro Gln Glu Arg His Ser Ile Arg Val Pro Glu Ser Gly Glu His Tyr
         275                 280                 285

Glu Leu His Leu Leu His Tyr Leu Gln Glu Asn Leu Gly Ser Arg Ile
         290                 295                 300

Ala Ala Leu Lys Val Ile
305                 310
```

```
<210>   4
<211>   1197
<212>   DNA
<213>   Homo sapiens

<400>   4
attttgaagg caccaaagac tcccctttag agcatcacct gtacgtagtc agttacgtaa
    60

atcctggaga ggtgacaagg ctgactgacc gtggctactc acattcttgc tgcatcagtc
    120

agcactgtga cttctttata agtaagtata gtaaccagaa gaatccacac tgtgtgtccc
    180

tttacaagct atcaagtcct gaagatgacc caacttgcaa aacaaaggaa ttttgggcca
    240
```

1.ST25.txt

```
ccattttgga ttcagcaggt cctcttcctg actatactcc tccagaaatt ttctcttttg
   300

aaagtactac tggatttaca ttgtatggga tgctctacaa gcctcatgat ctacagcctg
   360

gaaagaaata tcctactgtg ctgttcatat atggtggtcc tcagggtcaa atagaaattg
   420

acgatcaggt ggaaggactc caatatctag cttctcgata tgatttcatt gacttagatc
   480

gtgtgggcat ccacggctgg tcctatggag gatacctctc cctgatggca ttaatgcaga
   540

ggtcagatat cttcagggtt gctattgctg gggccccagt cactctgtgg atcttctatg
   600

atacaggata cacggaacgt tatatgggtc accctgacca gaatgaacag ggctattact
   660

taggatctgt ggccatgcaa gcagaaaagt tcccctctga accaaatcgt ttactgctct
   720

tacatggttt cctggatgag aatgtccatt ttgcacatac cagtatatta ctgagttttt
   780

tagtgagggc tggaaagcca tatgatttac agatctatcc tcaggagaga cacagcataa
   840

gagttcctga atcgggagaa cattatgaac tgcatctttt gcactacctt caagaaaacc
   900

ttggatcacg tattgctgct ctaaaagtga tataattttg acctgtgtag aactctctgg
   960

tatacactgg ctatttaacc aaatgaggag gtttaatcaa cagaaaacac agaattgatc
  1020

atcacatttt gatacctgcc atgtaacatc tactcctgaa aataaatgtg gtgccatgca
  1080

ggggttatcgg atttgtgctc gtaatctaat accttaagct cacatgttca aaatcacttt
  1140

atacatattc ctgagagacc cagcaatacc ataagaatta ctaaaaaaaa aaaaaaa
  1197
```

<210>   5
<211>   465
<212>   PRT
<213>   Homo sapiens

1.ST25.txt

<400>   5

```
Thr Gly Thr Ala Asn Pro Lys Val Thr Phe Lys Met Ser Glu Ile Met
1               5                   10                  15

Ile Asp Ala Glu Gly Arg Ile Ile Asp Val Ile Asp Lys Glu Leu Ile
                20                  25                  30

Gln Pro Phe Glu Ile Leu Phe Glu Gly Val Glu Tyr Ile Ala Arg Ala
            35                  40                  45

Gly Trp Thr Pro Glu Gly Lys Tyr Ala Trp Ser Ile Leu Leu Asp Arg
        50                  55                  60

Ser Gln Thr Arg Leu Gln Ile Val Leu Ile Ser Pro Glu Leu Phe Ile
65                  70                  75                  80

Pro Val Glu Asp Asp Val Met Glu Arg Gln Arg Leu Ile Glu Ser Val
                85                  90                  95

Pro Asp Ser Val Thr Pro Leu Ile Ile Tyr Glu Glu Thr Thr Asp Ile
            100                 105                 110

Trp Ile Asn Ile His Asp Ile Phe His Val Phe Pro Gln Ser His Glu
        115                 120                 125

Glu Glu Ile Glu Phe Ile Phe Ala Ser Glu Cys Lys Thr Gly Phe Arg
    130                 135                 140

His Leu Tyr Lys Ile Thr Ser Ile Leu Lys Glu Ser Lys Tyr Lys Arg
145                 150                 155                 160

Ser Ser Gly Gly Leu Pro Ala Pro Ser Asp Phe Lys Cys Pro Ile Lys
            165                 170                 175

Glu Glu Ile Ala Ile Thr Ser Gly Glu Trp Glu Val Leu Gly Arg His
            180                 185                 190

Gly Ser Asn Ile Gln Val Asp Glu Val Arg Arg Leu Val Tyr Phe Glu
        195                 200                 205

Gly Thr Lys Asp Ser Pro Leu Glu His His Leu Tyr Val Val Ser Tyr
    210                 215                 220

Val Asn Pro Gly Glu Val Thr Arg Leu Thr Asp Arg Gly Tyr Ser His
225                 230                 235                 240

Ser Cys Cys Ile Ser Gln His Cys Asp Phe Phe Ile Ser Lys Tyr Ser
            245                 250                 255

Asn Gln Lys Asn Pro His Cys Val Ser Leu Tyr Lys Leu Ser Ser Pro
            260                 265                 270

Glu Asp Asp Pro Thr Cys Lys Thr Lys Glu Phe Trp Ala Thr Ile Leu
            275                 280                 285
```

1.ST25.txt

Asp Ser Ala Gly Pro Leu Pro Asp Tyr Thr Pro Pro Glu Ile Phe Ser
290   295   300

Phe Glu Ser Thr Thr Gly Phe Thr Leu Tyr Gly Met Leu Tyr Lys Pro
305   310   315   320

His Asp Leu Gln Pro Gly Lys Lys Tyr Pro Thr Val Leu Phe Ile Tyr
                325   330   335

Gly Gly Pro Gln Val Ala Ile Ala Gly Ala Pro Val Thr Leu Trp Ile
                340   345   350

Phe Tyr Asp Thr Gly Tyr Thr Glu Arg Tyr Met Gly His Pro Asp Gln
        355   360   365

Asn Glu Gln Gly Tyr Tyr Leu Gly Ser Val Ala Met Gln Ala Glu Lys
370   375   380

Phe Pro Ser Glu Pro Asn Arg Leu Leu Leu Leu His Gly Phe Leu Asp
385   390   395   400

Glu Asn Val His Phe Ala His Thr Ser Ile Leu Leu Ser Phe Leu Val
                405   410   415

Arg Ala Gly Lys Pro Tyr Asp Leu Gln Ile Tyr Pro Gln Glu Arg His
                420   425   430

Ser Ile Arg Val Pro Glu Ser Gly Glu His Tyr Glu Leu His Leu Leu
        435   440   445

His Tyr Leu Gln Glu Asn Leu Gly Ser Arg Ile Ala Ala Leu Lys Val
        450   455   460

Ile
465

<210>   6
<211>   1669
<212>   DNA
<213>   Homo sapiens

<400>   6
aacaggtaca gcaaatccta aagtcacttt tagatatcta caaataatca ttgatgatca
        60

aggaaggatc atagatgtca tagataagga actaattcaa ccttttgaga ttctatttga
        120

aggagttgaa tatattgcca gagctggatg gactcctgag ggaaaatatg cttggtccat
        180

cctactagat cgctcccaga ctcgcctaca gatagtgttg atctcacctg aattatttat
        240

1.ST25.txt

cccagtagaa gatgatgtta tggaaaggca gagactcatt gagtcagtgc ctgattctgt
300

gacgccacta attatctatg aagaaacaac agacatctgg ataaatatcc atgacatctt
360

tcatgttttt ccccaaagtc acgaagagga aattgagttt atttttgcct ctgaatgcaa
420

aacaggtttc cgtcatttat acaaaattac atctatttta aaggaaagca aatataaacg
480

atccagtggt gggctgcctg ctccaagtga tttcaagtgt cctatcaaag aggagatagc
540

aattaccagt ggtgaatggg aagttcttgg ccggcatgga tctaatatcc aagttgatga
600

agtcagaagg ctggtatatt ttgaaggcac caaagactcc cctttagagc atcacctgta
660

cgtagtcagt tacgtaaatc ctggagaggt gacaaggctg actgaccgtg gctactcaca
720

ttcttgctgc atcagtcagc actgtgactt ctttataagt aagtatagta accagaagaa
780

tccacactgt gtgtcccttt acaagctatc aagtcctgaa gatgacccaa cttgcaaaac
840

aaaggaattt tgggccacca ttttggattc agcaggtcct cttcctgact atactcctcc
900

agaaattttc tcttttgaaa gtactactgg atttacattg tatgggatgc tctacaagcc
960

tcatgatcta cagcctggaa agaaatatcc tactgtgctg ttcatatatg gtggtcctca
1020

ggttgctatt gctggggccc cagtcactct gtggatcttc tatgatacag gatacacgga
1080

acgttatatg ggtcaccctg accagaatga acaggctat tacttaggat ctgtggccat
1140

gcaagcagaa aagttccct ctgaaccaaa tcgtttactg ctcttacatg gttcctgga
1200

tgagaatgtc cattttgcac ataccagtat attactgagt ttttagtga gggctggaaa
1260

gccatatgat ttacagatct atcctcagga gagacacagc ataagagttc ctgaatcggg
1320

agaacattat gaactgcatc ttttgcacta ccttcaagaa aaccttggat cacgtattgc

1.ST25.txt

1380

tgctctaaaa gtgatataat tttgacctgt gtagaactct ctggtataca ctggctattt
1440

aaccaaatga ggaggtttaa tcaacagaaa acacagaatt gatcatcaca ttttgatacc
1500

tgccatgtaa catctactcc tgaaaataaa tgtggtgcca tgcaggggtc tacggtttgt
1560

ggtagtaatc taatacctta accccacatg ctcaaaatca aatgatacat attcctgaga
1620

gacccagcaa taccataaga attactaaaa aaaaaaaaaa aaaaaaaaa
1669

<210>   7
<211>   360
<212>   PRT
<213>   Homo sapiens

<400>   7

Glu Glu Asp Ala Arg Ser Ala Gly Val Ala Thr Phe Val Leu Gln Glu
1   5   10   15

Glu Phe Asp Arg Tyr Ser Gly Tyr Trp Trp Cys Pro Lys Ala Glu Thr
                20   25   30

Thr Pro Ser Gly Gly Lys Ile Leu Arg Ile Leu Tyr Glu Glu Asn Asp
            35   40   45

Glu Ser Glu Val Glu Ile Ile His Val Thr Ser Pro Met Leu Glu Thr
        50   55   60

Arg Arg Ala Asp Ser Phe Arg Tyr Pro Lys Thr Gly Thr Ala Asn Pro
65   70   75   80

Lys Val Thr Phe Lys Met Ser Glu Ile Met Ile Asp Ala Glu Gly Arg
                85   90   95

Ile Ile Val Asp Glu Val Arg Arg Leu Val Tyr Phe Glu Gly Thr Lys
                100   105   110

Asp Ser Pro Leu Glu His His Leu Tyr Val Val Ser Tyr Val Asn Pro
                115   120   125

Gly Glu Val Thr Arg Leu Thr Asp Arg Gly Tyr Ser His Ser Cys Cys
            130   135   140

Ile Ser Gln His Cys Asp Phe Phe Ile Ser Lys Tyr Ser Asn Gln Lys
145   150   155   160

1.ST25.txt

```
Asn Pro His Cys Val Ser Leu Tyr Lys Leu Ser Ser Pro Glu Asp Asp
            165             170                 175
Pro Thr Cys Lys Thr Lys Glu Phe Trp Ala Thr Ile Leu Asp Ser Ala
            180             185                 190
Gly Pro Leu Pro Asp Tyr Thr Pro Pro Glu Ile Phe Ser Phe Glu Ser
            195             200                 205
Thr Thr Gly Phe Thr Leu Tyr Gly Met Leu Tyr Lys Pro His Asp Leu
    210             215             220
Gln Pro Gly Lys Lys Tyr Pro Thr Val Leu Phe Ile Tyr Gly Gly Pro
225             230             235             240
Gln Val Gln Leu Val Asn Asn Arg Phe Lys Gly Val Lys Tyr Phe Arg
            245             250             255
Leu Asn Thr Leu Ala Ser Leu Gly Tyr Val Val Val Val Ile Asp Asn
            260             265             270
Arg Gly Ser Cys His Arg Gly Leu Lys Phe Glu Gly Ala Phe Lys Tyr
            275             280             285
Lys Met Gly Gln Ile Glu Ile Asp Asp Gln Val Glu Gly Leu Gln Tyr
    290             295             300
Leu Ala Ser Arg Tyr Asp Phe Ile Asp Leu Asp Arg Val Gly Ile His
305             310             315             320
Gly Trp Ser Tyr Gly Gly Tyr Leu Ser Leu Met Ala Leu Met Gln Arg
                325             330             335
Ser Asp Ile Phe Arg Val Ala Ile Ala Gly Ala Pro Val Thr Leu Trp
            340             345             350
Ile Phe Tyr Asp Thr Gly Tyr Thr
            355             360
```

```
<210>   8
<211>   1083
<212>   DNA
<213>   Homo sapiens

<400>   8
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    60
atattctggc tattggtggt gtccaaaagc tgaaacaact cccagtggtg gtaaaattct
    120
tagaattcta tatgaagaaa atgatgaatc tgaggtggaa attattcatg ttacatcccc
    180
tatgttggaa acaaggaggg cagattcatt ccgttatcct aaaacaggta cagcaaatcc
```

1.ST25.txt

240

taaagtcact tttaagatgt cagaaataat gattgatgct gaaggaagga tcatagttga
300

tgaagtcaga aggctggtat attttgaagg caccaaagac tcccctttag agcatcacct
360

gtacgtagtc agttacgtaa atcctggaga ggtgacaagg ctgactgacc gtggctactc
420

acattcttgc tgcatcagtc agcactgtga cttctttata agtaagtata gtaaccagaa
480

gaatccacac tgtgtgtccc tttacaagct atcaagtcct gaagatgacc caacttgcaa
540

aacaaaggaa ttttgggcca ccattttgga ttcagcaggt cctcttcctg actatactcc
600

tccagaaatt ttctcttttg aaagtactac tggatttaca ttgtatggga tgctctacaa
660

gcctcatgat ctacagcctg gaaagaaata tcctactgtg ctgttcatat atggtggtcc
720

tcaggtgcag ttggtgaata atcggtttaa aggagtcaag tatttccgct tgaataccct
780

agcctctcta ggttatgtgg ttgtagtgat agacaacagg ggatcctgtc accgagggct
840

taaatttgaa ggcgccttta aatataaaat gggtcaaata gaaattgacg atcaggtgga
900

aggactccaa tatctagctt ctcgatatga tttcattgac ttagatcgtg tgggcatcca
960

cggctggtcc tatggaggat acctctccct gatggcatta atgcagaggt cagatatctt
1020

cagggttgct attgctgggg ccccagtcac tctgtggatc ttctatgata caggatacac
1080

gga
1083